MBHB CASE NO. 99,274-F

Title: Isolated Laminn 10

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ISOLATED LAMININ 10

Cross Reference

This application claims priority to U. S. provisional application serial no. 60/257,449, filed December 21, 2000; U. S. provisional application serial no. 60/279,282, filed March 28, 2001; U. S. provisional application serial no. to be assigned, filed November 13, 2001.

Field of the Invention

This application relates to cell biology, molecular biology, proteins, nucleic acids, and laminins.

Background of the Invention

Basal laminae (basement membranes) are sheet-like, cell-associated extracellular matrices that play a central role in cell growth, tissue development, and tissue maintenance. They are present in virtually all tissues, and appear in the earliest stages of embryonic development.

Basal laminae are central to a variety of architectural and cell-interactive functions (See for example, Malinda and Kleinman, Int. J. Biochem. Cell Biol. 28:957-959 (1996); Aumailley and Krieg, J. Invest. Dermatology 106:209-214 (1996)). For example:

1. They serve as architectural supports for tissues, providing adhesive substrata for cells.
2. They create perm-selective barriers between tissue compartments that impede the migration of cells and passively regulate the exchange of macromolecules. These properties are illustrated by the kidney glomerular basement membrane, which functions as an important filtration structure, creating an effective blood-tissue barrier that is not permeable to most proteins and cells.

3. Basal laminae create highly interactive surfaces that can promote cell migration and cell elongation during embryogenesis and wound repair. Following an injury, they provide a surface upon which cells regenerate to restore normal tissue function.

4. Basal laminae present information encoded in their structure to contacting cells that is important for differentiation and tissue maintenance. This information is communicated to the cells through various receptors that include the integrins, dystroglycan, and cell surface proteoglycans. Signaling is dependent not only on the presence of matrix ligands and corresponding receptors that interact with sufficient affinities, but also on such topographical factors as ligand density in a three-dimensional matrix "landscape", and on the ability of basal lamina components to cluster receptors. Because these matrix proteins can be long-lived, basal laminae create a "surface memory" in the basal lamina for resident and transient cells.

The basal lamina is largely composed of laminin and type IV collagen heterotrimers that in turn become organized into complex polymeric structures. To date, six type IV collagen chains and at least twelve laminin subunits have been identified. These chains possess shared and unique functions and are expressed with specific temporal (developmental) and spatial (tissue-site specific) patterns.

Laminins are a family of heterotrimeric glycoproteins that reside primarily in the basal lamina. They function via binding interactions with neighboring cell receptors, and by forming laminin networks, and they are important signaling molecules that can strongly influence cellular function. Laminins are important in both maintaining cell/tissue phenotype as well as promoting cell growth and differentiation in tissue repair and development.

Laminins are large, multi-domain proteins, with a common structural organization. The laminin molecule integrates various matrix and cell interactive functions into one molecule.

A laminin molecule is comprised of an α-, β-, and γ-chain subunit joined together through a coiled-coil domain. Within this structure are identifiable domains that possess binding activity towards other laminin and basal lamina molecules, and

membrane-bound receptors. Domains VI, IVb, and IVa form globular structures, and domains V, IIIb, and IIIa (which contain cysteine-rich EGF-like elements) form rod-like structures (Kamiguchi et al., Ann. Rev. Neurosci. 21:97-125 (1998)). Domains I and II of the three chains participate in the formation of a triple-stranded coiled-coil structure (the long arm).

Table 1 shows the individual chains that each laminin type is composed of:

TABLE 1. Known laminin family members

Protein	*Chains*
Laminin-1	α1β1γ1
Laminin-2	α2β1γ1
Laminin 3	α1β2γ1
Laminin-4	α2β2γ1
Laminin-5	α3β3γ2
Laminin-6	α3β1γ1
Laminin-7	α3β2γ1
Laminin-8	α4β1γ1
Laminin-9	α4β2γ1
Laminin-10	α5β1γ1
Laminin-11	α5β2γ1
Laminin-12	α2β1γ3

Four structurally-defined family groups of laminins have been identified. The first group of five identified laminin molecules, including laminin 10 all share the β1 and γ1 chains, and vary by their α-chain composition (α1 to α5 chain). The second group of five identified laminin molecules all share the β2 and γ1 chain, and again vary by their α-chain composition. The third group of identified laminin molecules has one identified member, laminin 5, with a chain composition of α3β3γ2. The fourth group of identified laminin molecules has one identified member, laminin 12, with the newly identified γ3 chain (α2β1γ3).

Some progress has been made in elucidating the relationship between domain structure and function (See, for example, Wewer and Engvall, Neuromusc. Disord. 6:409-418 (1996)). The overall sequence similarity among the homologous domains in different chains varies, but it is highest in domain VI (thought to play a key role in laminin polymerization), followed by domains V (possibly involved in protein-protein interactions) and III (entactin/nidogen binding; possible cell adhesion sites), and is

lowest in domains I, II (both thought to be involved in intermolecular assembly, and containing possible cell adhesion sites), and G. Not all domains are present in all 3 types of chains. The globular G domain (thought to be involved in cell receptor binding) is present only in the α chains. Other domains may not be present in all chains within a certain chain type. For example, domain VI is absent from α3, α4, and γ2 chains (Wewer and Engvall, 1996).

As a result of their large size (>600 kD) and unique structure, the laminin molecules can be resolved in the electron microscope (Wewer and Engvall, 1996). Typically, laminins appear as cross-shaped molecules in an EM. The three short arms of the cross represent the amino terminal portions of each of the three separate laminin chains (one short arm per chain). The long arm of the cross is composed of the C-terminal parts of the three chains, which together form a coiled coil structure (Wewer and Engvall, 1996). The long arm ends with the globular G domain.

The coiled-coil domain of the long arm is crucial for assembly of the three chains of laminin (Yurchenco et al., Proc. Natl. Acad. Sci. 94:10189-10194 (1997)). Disulfide bonds bridge and stabilize all three chains in the most proximal region of the long arm and join the β and γ chains in the most distal region of the long arm.

A model of laminin receptor-facilitated self-assembly, based on studies conducted with cultured skeletal myotubes and Schwann cells, predicts that laminins bind to their receptors, which freely diffuse in a fluidic membrane, when ligand-free. Receptor engagement forces the laminins into a high local two-dimensional concentration, facilitating their mass-action driven assembly into ordered surface polymers. In this process, the engaged receptors are also reorganized, accompanied by cytoskeletal rearrangements (Colognato, J. Cell Biol. 145:619-631 (1999)). This reorganization activates the receptors, causing signal transduction with the alteration of cell expression, shape and/or behavior. The evidence is that laminins must possess both cell-interacting and architecture-forming sites, which are located in different protein domains and on different subunits.

One class of laminin receptors are the integrins, which are cell surface receptors that mediate many cell-matrix and cell-cell interactions. Integrins are heterodimers, consisting of an α and a β subunit. 16 α- and 8 β-subunits are known, and at least 22

combinations of α and β subunits have been identified to date. Some integrins have only one or a few known ligands, whereas others appear to be very promiscuous. Binding to integrins is generally of low affinity, and is dependent on divalent cations. Integrins, activated through binding to their ligands, transduce signals via kinase activation cascades, such as focal adhesion and mitogen-activated kinases. Several different integrins bind different laminin isoforms more or less specifically (Aumailley et al., In The Laminins, Timpl and Ekblom, eds., Harwood Academic Publishers, Amsterdam. pp. 127-158 (1996)).

Laminin isoforms are expressed in tissue-specific and developmentally regulated patterns and they play significant roles in adhesion, migration, proliferation and differentiation of many cell types (Timpl, R., and Brown, J.C. (1994) *Matrix Biol.* 14(4), 275-81.; Ekblom, P., Timpl, R. (ed) (1996) *The laminins* Vol. 2. Cell Adhesion & Communication. Edited by Goridis, C., Harwood Academic Publishers GmbH, Amsterdam; Sorokin, L. M., et al. (1997) *Dev.Biol.* 189(2), 285-300.; Aumailley, M., and Smyth, N. (1998) *J. Anat.* 193(Pt 1), 1-21).

The laminin α5 chain, a component of laminin-10 (α5β1γ1) and laminin-11 (α5β2γ1), is expressed widely in adult tissues including placenta, heart, lung, skeletal muscle, kidney, and pancreas (Sorokin, L. M., et al. (1997) *Dev. Biol.* 189(2), 285-300; Patton, B. L., et al. (1997) *J. Cell Biol.* 139(6), 1507-21; Miner, J. H., et al. (1997) *J. Cell Biol.* 137(3), 685-701; Miner, J. H., et al. (1995) J. Biol. Chem. 270(48), 28523-6; Sorokin, L. M., et al. (1997) Dev. *Dyn.* 210(4), 446-62). Embryos lacking laminin α5 exhibit several developmental abnormalities, such as exencephaly and syndactyly, as well as dysmorphogenesis of the placental labyrinth and die late in embryogenesis (Miner, J. H., et al. (2000) *Dev.* Biol. 217(2), 278-89; Miner, J. H., et al. (1998) *J.Cell Biol.* 143(6), 1713-23). Laminin α5 chain-containing isoforms may therefore be important in placental endothelial cell migration and blood vessel branching, and in formation of proper basal laminae.

Integrin-mediated recognition of ECM molecules results in intracellular signaling that affects a range of cell behaviors (Clark, E. A. et al., Science 268(5208), 233-9 (1995)). In endothelial cells, these signals affect focal adhesions and cytoskeletal organization. Therefore, integrin-mediated endothelial cell recognition of laminin and

other BM molecules may determine cell-to-matrix adhesiveness and mediate signals that are essential for the maintenance and normal functioning of blood vessels (Davis, G. E. et al., Exp. Cell Res. 216(1), 113-23 (1995); Dejana, E. et al., Kidney Int. 43(1), 61-5 (1993); and Shattil, S.J. et al., J. Clin. Invest. 100(11 Suppl), S91-5 (1997)). Laminin-8 and laminin-10 are secreted by endothelial cells, and are major components of the subendothelial basement membrane (Sorokin, L.M. et al., Dev. Biol. 189(2), 285-300 (1997); Iivanainen, A. et al., J. Biol. Chem. 272(44), 27862-8 (1997); Patton, B. L. et al., J. Cell Biol. 139(6), 1507-21 (1997), Miner, J.H. et al., J. Cell Biol. 137(3), 685-701 (1997); Sorokin, L. et al., Eur. J. Biochem. 223(2), 603-10 (1994); and Tokida, Y. et al., J. Biol. Chem. 265(30), 18123-9 (1990)).

There have been no reports of isolated laminin 10 that is free of other laminin chains. Studies on the function of laminin-10 have frequently used commercial preparations, which are normally prepared using proteolytic digestion and subsequent immunoaffinity chromatography resulting in a truncated mixture of α5-chain containing laminin isoforms (Sixt, M. et al., J. Biol. Chem. 276(22), 18878-87 (2001)). Attempts to purify laminin 10 from cell sources by affinity chromatography using laminin chain antibodies have been unsuccessful in eliminating, for example, laminin β2 chain, which is a component of laminin 11. (See, for example, Sixt, M. et al., J. Biol. Chem. 276(22), 18878-87 (2001)) Thus, such preparations represent a mixture of laminin 10 and laminin 11.

Despite the broad tissue distribution of the laminin α5 chain and laminin 10, the full length human laminin α5 chain sequence is not known, nor is there a means to isolate laminin 10 away from other laminins, nor has a means for recombinant expression of laminin 10 previously been developed. Isolated laminin 10 would have numerous research and therapeutic purposes including, but are not limited to, treating injuries to vascular tissue, promoting cell attachment and migration, ex vivo cell therapy, improving the biocompatibility of medical devices, and preparing improved cell culture devices and media.

Thus, there is a need in the art for isolated laminin-10 for research and therapeutic purposes, and methods for making isolated laminin 10.

Summary of the Invention

In one aspect, the present invention provides an isolated nucleic acid encoding a full-length human laminin α5 chain consisting of the nucleic acid sequence of SEQ ID NO:1, or the complement thereof, as well as vectors comprising the sequence, and host cells transfected with such vectors. In another aspect, the present invention provides isolated laminin α5 chain protein consisting of the amino acid sequence of SEQ ID NO:2.

In another aspect, the present invention provides isolated laminin 10, and methods for producing isolated laminin 10. In a further aspect, the present invention provides recombinant host cells that express laminin 10 chains and secrete recombinant laminin 10.

In a further aspect, the present invention provides pharmaceutical compositions, comprising isolated laminin 10 together with a pharmaceutically acceptable carrier. Such pharmaceutical compositions can optionally be provided with other extracellular matrix components.

In another aspect, the present invention provides methods and kits for accelerating the healing of injuries to vascular tissue, and for improving the biocompatibility of grafts used for treating such injuries. In specific examples, laminin 10 or pharmaceutical compositions thereof are used to:

a. promote re-endothelialization at the site of vascular injuries;

b. improve the "take" of grafts;

c. improve the biocompatibility of medical devices; and/or

d. promote cell attachment and subsequent cell stasis, proliferation, differentiation, and/or migration.

by providing an amount effective of isolated laminin 10 for the various methods. In preferred embodiments of all of these methods, recombinant laminin 10 is used. The kits comprise an amount of isolated laminin 10, or pharmaceutical compositions thereof, effective for the desired effect, and instructions for the use thereof.

In a further aspect, the present invention provides improved medical devices and grafts, wherein the improvement comprises providing medical devices and grafts with an

amount effective of isolated laminin 10, or a pharmaceutical composition of the invention.

In a further aspect, the invention provides improved cell culture devices, and methods for preparing improved cell culture devices, for the growth and maintenance of cells in culture, by providing an amount effective to a cell culture device of isolated laminin 10 for cell attachment and subsequent cell stasis, proliferation, differentiation, and/or migration.

Brief Description of the Figures

Figure 1. cDNA-derived amino acid sequence of the human laminin 5 chain and alignment with the mouse chain. Upper sequence, human α5 chain; Lower sequence, mouse sequence. Domain boundaries are depicted, and adhesive tripeptide sequences RGD and LRE are boxed. The potential cleavage site of the signal peptide is indicated by a solid triangle (Predicted by PSORT II). The five possible polymorphisms are shown as bold italic characters above the human sequence. The sequence submitted to GenBank by the Human Genome Project (accession NM_005560 (May 30, 2001)) differed from the present sequence at a few sites, indicated above the sequence in the figure. At these sites, the present sequence did not differ from the incomplete genomic sequences reported by Celera.

Figure 2. Characterization of r-laminin-10 using SDS-PAGE. (A) Silver stain: Conditioned medium of triple-transfected HEK293 cells (CM) and recombinant purified laminin-10 (r-laminin-10), were analyzed by SDS-PAGE on 4-15% gradient gels under reducing conditions; (B) Immunoblot of CM and r-laminin-10 under reducing conditions: Separated proteins on 5% gels were transferred onto PVDF membranes followed by staining with mAbs against laminin α5 (15H5), β1 (DG10), γ1 (clone 22) and FLAG-M2.; (C) Silver stain and immunoblot of rLN-10 under non-reducing conditions: Separated proteins on 5% gels were visualized by silver staining or transferred onto

PVDF membranes followed by staining with mAbs against laminin α5 (15H5), β1 (DG10), γ1 (2E8). The positions of molecular size markers are shown.

Figure 3. Cell adhesion activity of recombinant laminin-10 (rLN-10) and other proteins. (A) HT-1080 cell adhesion to rLN-10, recombinant laminin-8 (rLN-8), laminin-1/nidogen complex (LN-1/Nd), and fibronectin (FN) coated at increasing concentrations.; (B) IBE cell adhesion to rLN-10, rLN-8, LN-1/Nd, and FN coated at increasing concentrations.; (C) HSVEC adhesion to rLN-10 and rLN-8 coated at 3 and 10 mg/ml, and LN-1/Nd, commercial laminin-10/11 (LN-10/11), collagen type IV (Col IV) and FN coated at 10 mg/ml. N.T. means not-tested. Bound cells were quantitated spectrophometrically using adhesion to BSA as blank. Error bars indicate S.D.

Figure 4. HT-1080 cell and HSVEC adhesion assays on laminins coated at 10 g/ml. Text under columns indicate the integrin subunit mAbs used or other added substances. Adhesion shown is relative to control, designated 100. Adhesion to BSA was designated zero. Error bars indicate S.D.

Figure 5. HSVEC migration (endothelialization) on plastic coated with laminin-10 and other proteins. Migration of HSVECs into the cell free area coated with BSA, laminin-1/ nidogen complex (LN-1/Nd), recombinant laminin-8 (rLN-8), recombinant laminin-10 (rLN-10), fibronectin (FN), commercial laminin-10/11 (LN-10/11), gelatin, and collagen type IV (Col IV) was measured. The distance covered by cells from three different donors. N.T. means not-tested.

Table 2: **Primers used in laminin 5 expression construct preparation.**
Upper row, forward primer; Lower row, reverse primer.

plasmid	primer	primer sequence
KBX3	KZK1	5'-gccaccatggcgaagcggctctg-3' (SEQ ID NO.:21)
	Ba3r	5'-aagggcaggatccactgggg-3' (SEQ ID NO.:22)
BBL3	Bam4	5'-ctactgcgaagctggctctt-3' (SEQ ID NO.:23)
	Bcl1r	5'-ccaggtggtcctgggtatc-3' (SEQ ID NO.:24)

BNK2'	Bcl2	5'-gcgacaactgcctcctctac-3' (SEQ ID NO.:25)
	Not4r	5'-agtgggttcccaaagaatcc-3' (SEQ ID NO.:26)
BNL12	Bpu1F	5'-cctctgtgacgagctcacg-3' (SEQ ID NO.:27)
	Not4r	5'-agtgggttcccaaagaatcc-3' (SEQ ID NO.:26)
D29D30l	D29	5'-gatgtgtcccttgtcagtgccat-3' (SEQ ID NO.:28)
	D30l	5'-tgtcgtgttcagccgcttgaggt-3' (SEQ ID NO.:29)
NSK5	Not3	5'-ctctcagtgccttccaacaac-3' (SEQ ID NO.:30)
	Sal4r	5'-ctgactgtcgaagctgatgc-3' (SEQ ID NO.:31)
SFK2	Sal5	5'-ggaggtggtcagcctctaca-3' (SEQ ID NO.:32)
	FLAG1	5'-ttacttgtcatcgtcgtccttgtagtcggcggctgggcag-3' (SEQ ID NO.:33)
SFL13	Sal5	5'-ggaggtggtcagcctctaca-3' (SEQ ID NO.:32)
	m19R	5'-aatggtgccagactcagg-3' (SEQ ID NO.:34)

Detailed Description of the Preferred Embodiments

All references, patents and patent applications are hereby incorporated by reference in their entirety.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

In one aspect, the present invention provides an isolated nucleic acid encoding a full length laminin α5 chain polypeptide consisting of the amino acid sequence of SEQ

ID NO:2. In a preferred embodiment, the isolated nucleic acid consists of the sequence of SEQ ID NO:1, the complement thereof, or the RNA expression product thereof.

In an additional aspect, the present invention provides an isolated nucleic acid comprising a nucleic acid sequence encoding the 2,743 N-terminal amino acids (SEQ ID NO:36) of the human laminin α5 chain, which has not previously been reported. In a preferred embodiment, the isolated nucleic acid consists of the sequence of SEQ ID NO:35, the complements thereof, or the RNA expression product thereof.

An used herein, an "isolated nucleic acid sequence" refers to a nucleic acid sequence that is free of gene sequences which naturally flank the nucleic acid in the genomic DNA of the organism from which the nucleic acid is derived (i.e., genetic sequences that are located adjacent to the gene for the isolated nucleic molecule in the genomic DNA of the organism from which the nucleic acid is derived). An "isolated" laminin α5 chain nucleic acid sequence according to the present invention may, however, be linked to other nucleotide sequences that do not normally flank the recited sequence, such as a heterologous promoter sequence, or other vector sequences. It is not necessary for the isolated nucleic acid sequence to be free of other cellular material to be considered "isolated", as a nucleic acid sequence according to the invention may be part of an expression vector that is used to transfect host cells (see below).

In another aspect, the present invention provides recombinant expression vectors comprising a full length laminin α5 chain nucleic acid sequence, or a nucleic acid sequence expressing the 2,743 N-terminal amino acids (SEQ ID NO:36) of the human laminin α5 chain. In one embodiment, the expression vectors comprise a nucleic acid encoding a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:36, operatively linked to a heterologous (i.e.: is not the naturally occurring α5 laminin chain promoter) promoter. In a preferred embodiment, the isolated nucleic acid consists of a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:35. A promoter and a laminin α5 chain nucleic acid sequence are "operatively linked" when the promoter is capable of driving expression of the laminin α5 chain DNA into RNA.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a

"plasmid", which refers to a circular double stranded DNA into which additional DNA segments may be cloned. Another type of vector is a viral vector, wherein additional DNA segments may be cloned into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors), are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In the present invention, the expression of the laminin polypeptide sequence is directed by the promoter sequences of the invention, by operatively linking the promoter sequences of the invention to the gene to be expressed. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably, as the plasmid is the most commonly used form of vector. However, the invention is intended to include other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The vector may also contain additional sequences, such as a polylinker for subcloning of additional nucleic acid sequences, or a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed, including but not limited to the SV40 and bovine growth hormone poly-A sites. Also contemplated as an element of the vector is a termination sequence, which can serve to enhance message levels and to minimize read through from the construct into other sequences. Additionally, expression vectors typically have selectable markers, often in the form of antibiotic resistance genes, that permit selection of cells that carry these vectors.

In a further embodiment, the present invention provides host cells transfected with the laminin α5 chain-expressing recombinant expression vectors disclosed herein. As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid of

the invention, such as a recombinant expression vector of the invention, has been introduced. Such cells may be prokaryotic, which can be used, for example, to rapidly produce a large amount of the expression vectors of the invention, or may be eukaryotic, for functional studies.

The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

The host cells can be transiently or stably transfected with one or more of the expression vectors of the invention. Such transfection of expression vectors into prokaryotic and eukaryotic cells can be accomplished via any technique known in the art, including but not limited to standard bacterial transformations, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. (See, for example, *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press; *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY).

In another aspect, the present invention provides an isolated full length human laminin α5 chain polypeptide consisting of amino acid sequence of SEQ ID NO:2. In a further embodiment, of this aspect, the invention provides an isolated polypeptide consisting of the 2,743 N-terminal amino acids (SEQ ID NO:36) of the human laminin α5 chain.

As used herein, an "isolated polypeptide" refers to a polypeptide that is substantially free of other proteins, including other laminin chains, and gel agents, such as polyacrylamide and agarose. In a preferred embodiment, the isolated laminin polypeptide is free of detectable contaminating laminin chains. Thus, the protein can either be isolated from natural sources, or recombinant protein can be isolated from the transfected host cells disclosed above.

In a further aspect, the invention provides methods for detecting the presence of the laminin α5 chain in a protein sample, comprising providing a protein sample to be screened, contacting the protein sample to be screened with an antibody against the 2,743 N-terminal amino acids (SEQ ID NO:36) of the human laminin α5 chain, or fragments thereof, and detecting the formation of antibody-antigen complexes.

The antibody can be either polyclonal or monoclonal, although monoclonal antibodies are preferred. As used herein, the term "protein sample" refers to any sample that may contain the laminin α5 chain, including but not limited to tissues and portions thereof, tissue sections, intact cells, cell extracts, purified or partially purified protein samples, bodily fluids, nucleic acid expression libraries. Accordingly, this aspect of the present invention is useful for a variety of purposes including, but not limited to, immunolocalization, immunofluorescence analysis, Western blot analysis, ELISAs, and nucleic acid expression library screening. In one embodiment, the techniques may determine only the presence or absence of the human laminin α5 chain. Alternatively, the techniques may be quantitative, and provide information about the relative amount of laminin α5 chain in the sample. For quantitative purposes, ELISAs are preferred.

Detection of immunocomplex formation between the human laminin α5 chain and antibodies or fragments thereof directed against an amino acid sequence of SEQ ID NO:36, or fragments thereof, can be accomplished by standard detection techniques. For example, detection of immunocomplexes can be accomplished by using labeled antibodies or secondary antibodies.

Antibodies can be made by well-known methods, such as described in Harlow and Lane, Antibodies; A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1988). For example, all or a portion of the amino acid sequence of SEQ ID NO:36, together with an appropriate adjuvant, can be injected into an animal in an amount and at intervals sufficient to elicit an immune response. Animals are bled at regular intervals, preferably weekly, to determine antibody titer. Polyclonal antibodies against laminin α5 chain can then be purified directly by passing serum collected from the animal through a column to which non-antigen-related proteins prepared from the same expression system without laminin α5 chain bound.

Monoclonal antibodies can be produced by obtaining spleen cells from the animal. (See Kohler and Milstein, Nature 256, 495-497 (1975)). In one example, monoclonal antibodies (mAb) of interest are prepared by immunizing inbred mice with peptide fragments of the amino acid sequence of SEQ ID NO:36. The mice are immunized by the IP or SC route in an amount and at intervals sufficient to elicit an immune response. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of by the intravenous (IV) route. Lymphocytes, from antibody positive mice are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner under conditions which will allow the formation of stable hybridomas. The antibody producing cells and fusion partner cells are fused in polyethylene glycol at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells and are screened for antibody production by an immunoassay such as solid phase immunoradioassay. Hybridoma cells from antibody positive wells are cloned by a technique such as the soft agar technique of MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

In yet another aspect, the invention provides methods for detecting the presence in a sample of nucleic acid sequences encoding the human laminin α5 chain, comprising providing a nucleic acid sample to be screened, contacting the sample with a nucleic acid probe consisting of the nucleic acid sequence of SEQ ID NO:35 or fragments thereof, and detecting complex formation.

As used herein, the term "sample" refers to any sample that may contain nucleic acid sequences encoding the human laminin α5 chain, including but not limited to tissues and portions thereof, tissue sections, intact cells, cell extracts, purified or partially purified nucleic acid samples, DNA libraries, and bodily fluids. Accordingly, this aspect of the present invention may be used to test for the presence of laminin α5 chain mRNA or DNA in these various samples by standard techniques including, but not limited to, in

situ hybridization, Northern blotting, Southern blotting, DNA library screening, polymerase chain reaction (PCR) or reverse transcription-PCR (RT-PCR). In one embodiment, the techniques may determine only the presence or absence of the nucleic acid of interest. Alternatively, the techniques may be quantitative, and provide information about the relative amount of the nucleic acid of interest in the sample. For quantitative purposes, quantitative PCR and RT-PCR are preferred. Thus, in one example, RNA is isolated from a sample, and contacted with an oligonucleotide derived from the nucleic acid sequence of SEQ ID NO:35, or its complement, together with reverse transcriptase under suitable buffer and temperature conditions to produce cDNAs from the laminin α5 chain RNA. The cDNA is then subjected to PCR using primer pairs derived from the nucleic acid sequence of interest. For detecting laminin α5 chain nucleic acid sequences, standard labeling techniques can be used to label the probe, the nucleic acid of interest, or the complex between the probe and the nucleic acid of interest, including, but not limited to radio-, enzyme-, chemiluminescent-, or avidin or biotin-labeling techniques, all of which are well known in the art. (See, for example, *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA)).

In another aspect, the present invention provides isolated laminin 10. As used herein "laminin 10" encompasses both r-laminin 10 and heterotrimeric laminin 10 from naturally occurring sources. In a preferred embodiment, the laminin 10 comprises recombinant laminin 10 (or "r-laminin 10").

As used herein, the term "r-laminin 10" refers to recombinant heterotrimeric laminin 10, expressed by a host cell that has been transfected with one or more expression vectors comprising at least one nucleic acid sequence encoding a laminin 10 chain selected from the α5, β1 and γ1 chains, or processed/secreted forms thereof. Such r-laminin 10 can thus comprise α5, β1, and γ1 sequences from a single organism, or from different organisms. Various laminin 10 chain DNA sequences are known in the art, and the use of each to prepare the r-laminin 10 of the invention is contemplated. (See, for example, Pouliot, N. et al., Experimental Cell Research 261(2):360-71, (2000); Kikkawa,

Y. et al., Journal of Cell Science 113 (Pt 5):869-76, (2000); Church, HJ. et al., Biochemical Journal 332 (Pt 2):491-8, (1998); Sorokin, LM. et al., Developmental Biology 189(2):285-300, (1997); Miner, JH. et al., Journal of Biological Chemistry 270(48):28523-6, (1995); Sorokin, L. et al., European Journal of Biochemistry 223(2):603-10, (1994); all references incorporated by reference herein in their entirety). In a preferred embodiment, the r-laminin 10 comprises recombinant human α5, β1, and γ1 polypeptide chains.

As used herein, "isolated" means that the laminin 10 is substantially free of other proteins, including the laminin β2 chain polypeptide chain, and gel agents, such as polyacrylamide and agarose. In a preferred embodiment, the isolated laminin 10 is free of detectable laminin β2 polypeptide chains.

The invention encompasses those laminin molecules wherein one or two chains that make up the recombinant heterotrimeric laminin 10 are encoded by endogenous laminin 10 chains. In a preferred embodiment, each of the α5, β1, and γ1 polypeptide chains are expressed recombinantly.

Laminin 10 is a secreted protein, which is capable of being directed to the endoplasmic reticulum (ER), secretory vesicles, and the extracellular space as a result of a signal sequence. If the secreted protein is released into the extracellular space, the secreted protein can undergo extracellular processing to produce a "mature" protein. Such processing event can be variable, and thus may yield different versions of the final "mature protein". The isolated laminin 10 of the present invention includes heterotrimers comprising both the full length and any such processed laminin 10 polypeptide chains.

As used herein, a laminin 10 polypeptide chain refers to a polypeptide chain according to one or more of the following:

(a) comprises a polypeptide structure selected from the group consisting of:

1. R1-R2-R3
2. R1-R2-R3(e)
3. R3
4. R3(e)
5. R1-R3
6. R1-R3(e)

7. R2-R3

8. R2-R3(e)

wherein R1 is an amino terminal methionine; R2 is a signal sequence that is capable of directing secretion of the polypeptide, wherein the signal sequence may be the natural signal sequence for the particular laminin chain, that of another secreted protein, or an artificial sequence; R3 is a secreted laminin chain selected from group consisting of the α5, β1, and γ1 chains; and R3(e) is a secreted laminin chain selected from the α5, β1, and γ1 chains that further comprises an epitope tag (such as those described below), which can be placed at any position within the laminin chain amino acid sequence; and/or

(b) is encoded by a polynucleotide that hybridizes under high or low stringency conditions to the coding regions, or portions thereof, of one or more of the recombinant laminin 10 chain DNA sequences disclosed herein (SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, and SEQ ID NO:35), or complementary sequences thereof; and/or

(c) has at least 70% identity to one or more of the disclosed laminin 10 polypeptide chain amino acid sequences (SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, and SEQ ID NO:36), preferably at least 80% identity, and most preferably at least about 90% identity.

"Stringency of hybridization" is used herein to refer to conditions under which nucleic acid hybrids are stable. The invention also includes nucleic acids that hybridize under high stringency conditions (as defined herein) to all or a portion of the coding sequences of the laminin chain polynucleotides disclosed herein, or their complements. The hybridizing portion of the hybridizing nucleic acids is typically at least 50 nucleotides in length. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (T_M) of the hybrids. T_M decreases approximately 1-1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of

varying, but higher, stringency. Reference to hybridization stringency relates to such washing conditions. Thus, as used herein, high stringency refers to an overnight incubation at 42° C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

Also contemplated are laminin 10-encoding nucleic acid sequences that hybridize to the polynucleotides of the present invention at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH_2PO_4; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 ug/ml salmon sperm blocking DNA; followed by washes at 50°C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC).

Note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

As used herein, "percent identity" of two amino acids or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990), modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12, to determine nucleotide sequences identity to the nucleic acid molecules of the invention.

BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3, to determine an amino acid sequence identity to a polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al. (Nucleic Acids. Res. 25:3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. See http://www.ncbi.nlm.nih.gov.

Further embodiments of the present invention include polynucleotides encoding laminin 10 chain polypeptides having at least 70% identity, preferably at least 80% identity, and most preferably at least 90% identity to one or more of the polypeptide sequences contained in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, and SEQ ID NO:36 .

As used herein, "α5 polynucleotide" refers to polynucleotides encoding an laminin α5 chain of the same name. Such polynucleotides can be characterized by one or more of the following: (a) polynucleotides that encode polypeptides which share at least 70% identity, preferably 80% identity, and most preferably at least 90% identity with one or more sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO:4; (b) the α5 polynucleotides hybridize under low or high stringency conditions to one or more coding sequences selected from the group consisting of SEQ ID NO: 1 or SEQ ID NO:3; complementary sequences thereof; or (c) the α5 polynucleotides encode a laminin α5 chain polypeptide with a general structure selected from the group consisting of (1) R1-R2-R3; (2) R1-R2-R3(e); (3) R3; (4) R3(e); (5) R1-R3; (6) R1-R3(e); (7) R2-R3; and (8) R2-R3(e); wherein R1 and R2 are as described above, and R3 and R3(e) are as described above but comprise secreted α5 chain polypeptides.

As used herein, "β1 polynucleotides" refers to polynucleotides encoding a β1 laminin chain of the same name. Such polynucleotides can be characterized by one or more of the following: (a) polynucleotides that encode polypeptides which share at least 70% identity, preferably at least 80%, and most preferably at least 90% identity with one or more of the sequences selected from the group consisting of SEQ ID NO: 6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12; (b) the β1 polynucleotides hybridize under low or high stringency conditions to one or more coding sequences selected from the group

consisting of SEQ ID NO: 5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or complementary sequences thereof; or (c) the β1 polynucleotides encode a polypeptide with a general structure selected from (1) R1-R2-R3; (2) R1-R2-R3(e); (3) R3; (4) R3(e); (5) R1-R3; (6) R1-R3(e); (7) R2-R3; and (8) R2-R3(e); wherein R1 and R2 are as described above, and R3 and R3(e) are as described above but comprise secreted β1 chain polypeptides.

As used herein, "γ1 polynucleotides" refers to polynucleotides encoding a γ1 laminin chain of the same name. Such polynucleotides can be characterized by one or more of the following: (a) polynucleotides that encode polypeptides which share at least 70% identity, preferably at least 80%, and most preferably at least 90% identity with one or more of the sequences selected from the group consisting of SEQ ID NO: 14, SEQ ID NO:16, SEQ ID NO:18, or SEQ ID NO:20; (b) the γ1 polynucleotides hybridize under low or high stringency conditions to one or more of the coding sequence selected from the group consisting of SEQ ID NO: 13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19 or complementary sequences thereof; or (c) the γ1 polynucleotides encode a polypeptide with a general structure selected from (1) R1-R2-R3; (2) R1-R2-R3(e); (3) R3; (4) R3(e); (5) R1-R3; (6) R1-R3(e); (7) R2-R3; and (8) R2-R3(e); wherein R1 and R2 are as described above, and R3 and R3(e) are as described above but comprise secreted γ1 chain polypeptides.

As used herein, the term "epitope tag" refers to a polypeptide sequence that is expressed as part of a chimeric protein, where the epitope tag serves as a recognition site for binding of antibodies generated against the epitope tag, or for binding of other molecules that can be used for affinity purification of sequences containing the tag.

In a preferred embodiment, cDNAs encoding the laminin α5, β1 and γ1 chains, or fragments thereof, are subcloned into an expression vector. Alternatively, laminin α5, β1 and/or γ1 gene sequences, including one or more introns, can be used for sub-cloning into an expression vector.

In another aspect, the present invention provides laminin 10 expressing-cells that have been transfected with an expression vector containing promoter sequences that are operatively linked to nucleic acid sequences encoding at least one polypeptide sequence comprising a sequence selected from the group consisting of the α5, β1 and γ1 chains of

laminin 10, wherein the transfected cells secrete heterotrimeric laminin 10 containing the recombinant laminin chain. In a preferred embodiment, the cells are systematically transfected with recombinant expression vectors containing promoter sequences that are operatively linked to nucleic acid sequences encoding polypeptide sequences comprising the α5, β1 and γ1 chains of laminin 10, even more preferably, all human chains. After the multiple transfections, the cells express recombinant laminin 10 chains, which form the heterotrimeric r-laminin 10.

Transfection of the expression vectors into eukaryotic cells can be accomplished via any technique known in the art, including but not limited to calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. Transfection of bacterial cells can be done by standard methods.

In a preferred embodiment, the cells are stably transfected. Methods for stable transfection and selection of appropriate transfected cells are known in the art. In another preferred embodiment, a CMV promoter driven expression vector is used in a human kidney embryonic 293 cell line.

Any cell capable of expressing and secreting the r-laminin 10 can be used. Preferably, eukaryotic cells are used, and most preferably mammalian cells are used, including but not limited to kidney and epithelial cell lines. The promoter sequence used to drive expression of the individual chains or r-laminin 10 may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). Carbohydrate and disulfide post-translational modifications are believed to be required for laminin 10 protein folding and function. This makes the use of eukaryotic cells preferable for producing functional r-laminin 10, although other systems are useful for obtaining, for example, antigens for antibody production. In a most preferred embodiment, the mammalian cells do not express the laminin β2 chain endogenously. In another preferred embodiment, the cells do not express all of the laminin 10 chains endogenously.

The protein may comprise additional sequences useful for promoting purification of the protein, such as epitope tags and transport signals. Examples of such epitope tags

include, but are not limited to FLAG (Sigma Chemical, St. Louis, MO), myc (9E10) (Invitrogen, Carlsbad, CA), 6-His (Invitrogen; Novagen, Madison, WI), and HA (Boehringer Manheim Biochemicals). Examples of such transport signals include, but are not limited to, export signals, secretory signals, nuclear localization signals, and plasma membrane localization signals.

In one embodiment, at least one of the laminin chain polypeptide sequences, or fragments thereof, is operatively linked to a nucleic acid sequence encoding an "epitope tag", so that at least one of the chains is expressed as a fusion protein with an expressed epitope tag. The epitope tag may be expressed as the amino terminus, the carboxy terminus, or internal to any of the polypeptide chains comprising r-laminin 10, so long as the resulting r-laminin 10 remains functional.

In another embodiment, one of the r-laminin 10 chains is expressed as a fusion protein with a first epitope tag, and at least one other r-laminin chain is expressed as a fusion protein with a second epitope tag. This permits multiple rounds of purification to be carried out. Alternatively, the same epitope tag can be used to create fusion proteins with more than one of the r-laminin chains.

In a further embodiment, the epitope tag can be engineered to be cleavable from the r-laminin 10 chain(s). Alternatively, no epitope tag is fused to any of the r-laminin 10 chains, and the r-laminin 10 is isolated by standard techniques, including but not limited to affinity chromatography using laminin 10 specific antibodies or other laminin 10 binding molecules.

Media from cells transfected with a single laminin chain are initially analyzed on Western blots using laminin chain-specific antibodies. The expression of single laminin chains following transfection is generally intracellular. Clones showing reactivity against individual transfected chain(s) are verified by any appropriate method, such as PCR, reverse transcription-PCR, or nucleic acid hybridization, to confirm incorporation of the transfected gene. Preferably, analysis of genomic DNA preparations from such clones is done by PCR using laminin chain-specific primer pairs. Media from transfected clones producing all three chains are further analyzed for r-laminin 10 secretion and/or activity, by any appropriate method, including Western blot analysis and cell binding assays. Activity of the r-laminin 10 is preferably analyzed in a cell adhesion assay.

In a preferred embodiment, purification of r-laminin 10 is accomplished by passing media from the transfected cells through an antibody affinity column. In one embodiment, antibodies against a peptide epitope expressed on at least one of the recombinant chains are attached to an affinity column, and bind the r-laminin 10 that has been secreted into the media. The r-laminin 10 is removed from the column by passing excess peptide over the column. Eluted fractions are analyzed by any appropriate method, including gel electrophoresis and Western blot analysis. In a further embodiment, the peptide epitope can be cleaved after purification. In other embodiments, two or three separate r-laminin chains are expressed as fusion proteins, each with a different epitope tag, permitting two or three rounds of purification and a doubly or triply isolated r-laminin 10. The epitope tag can be engineered so as to be cleavable from the r-laminin 10 chain(s) after purification. Alternatively, no epitope tag is fused to any of the r-laminin 10 chains, and the r-laminin 10 is isolated by standard techniques, including but not limited to affinity chromatography using laminin 10 specific antibodies or other laminin 10 binding molecules.

The laminin 10 polypeptide chains of the present invention also include (i) substitutions with one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) substitution with one or more amino acid residues having substituents groups, or (iii) fusion of the mature polypeptide with another compound, such as a compound to increase the stability and/or solubility of the polypeptide (for example, polyethylene glycol), or (iv) fusion of the polypeptide with additional amino acids, such as an IgG Fc fusion region peptide, or leader or secretory sequence, or a sequence facilitating purification. Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

For example, polypeptide variants containing amino acid substitutions of charged amino acids with other charged or neutral amino acids may produce proteins with improved characteristics, such as less aggregation. Aggregation of pharmaceutical formulations both reduces activity and increases clearance due to the aggregate's immunogenic activity. (Pinckard et al., Clin. Exp. Immunol. 2:331-340 (1967); Robbins et al., Diabetes 36: 838-845 (1987); Cleland et al., Crit. Rev. Therapeutic Drug Carrier Systems 10:307-377 (1993).)

The present invention further provides pharmaceutical compositions comprising isolated laminin 10 and a pharmaceutically acceptable carrier. In a preferred embodiment, the pharmaceutical composition comprises isolated r-laminin 10. According to this aspect of the invention, other agents can be included in the pharmaceutical compositions, depending on the condition being treated. The pharmaceutical composition may further comprise one or more other compounds, including but not limited to any of the collagens, other laminin types, fibronectin, vitronectin, cadherins, integrins, α-dystroglycan, entactin/nidogen, α-dystroglycan, glycoproteins, proteoglycans, heparan sulfate proteoglycan, glycosaminoglycans, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, or nerve growth factors, and peptide fragments thereof.

Pharmaceutical preparations comprising isolated laminin 10 can be prepared in any suitable form, and generally comprise the isolated laminin 10 in combination with any of the well known pharmaceutically acceptable carriers. The carriers can be injectable carriers, topical carriers, transdermal carriers, and the like. The preparation may advantageously be in a form for topical administration, such as an ointment, gel, cream, spray, dispersion, suspension or paste. The preparations may further advantageously include preservatives, antibacterials, antifungals, antioxidants, osmotic agents, and similar materials in composition and quantity as is conventional. Suitable solutions for use in accordance with the invention are sterile, are not harmful for the proposed application, and may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc. For assistance in formulating the compositions of the present invention, one may refer to Remington's Pharmaceutical Sciences, 15th Ed., Mack Publishing Co., Easton, Pa. (1975).

In further aspect, the present invention provides methods and kits comprising isolated laminin 10, or pharmaceutical compositions thereof (and instructions for using the isolated laminin 10 in the kits) for accelerating the healing of injuries to vascular tissue, and for improving the biocompatibility of grafts used for treating such injuries. In a preferred embodiment of each of the methods disclosed below, isolated laminin 10 is

used. In specific examples, isolated laminin 10, isolated r-laminin 10, or pharmaceutical compositions thereof are used to:

a. promote re-endothelialization at the site of vascular injuries;

b. improve the "take" of grafts;

c. improve the biocompatibility of medical devices;

d. promote cell attachment and subsequent cell stasis, proliferation, differentiation, and/or migration

by providing an amount effective of isolated laminin 10 or pharmaceutical compositions thereof for the various methods.

Endothelial cells normally rest on a subendothelium, composed of collagen type I/III, elastin, fibronectin, glycosaminoglycans, and a basement membrane, mainly consisting of laminin and type IV collagen. Several of the invasive treatments used in the repair of vascular occlusive diseases, or the disease itself, may cause large areas of endothelial cell-denudation in the vessel wall. Endothelialization is, in part, dependent upon the underlying matrix, as subendothelial proteins have been shown to be important modulators of endothelial cell function (Madri, J.A. et al., Am. J. Pathol. 132(1), 18-27 (1988); and Madri, J.A. et al., J. Cell Biochem. 45(2), 123-30 (1991). In an effort to enhance endothelialization of grafts in humans, extensive research has been devoted to identifying substances that promote endothelial cell migration. Endothelial cell migration is a key element of endothelialization of vascular grafts, whether by anastomotic ingrowth, transmural capillary ingrowth, adherence of circulating cells, or *ex vivo* cell seeding. Ultimately, it is desirable to encourage spontaneous migration *in vivo* because this would minimize the need for *ex vivo* graft and cell manipulation. In addition, retention of cells on the vascular surface is necessary prior to migration (Dixit, P. et al., J. Biomed. Mater. Res. 56(4), 545-55 (2001)). Pretreatment of the graft with an adhesive substrate significantly enhances endothelial cell attachment to graft samples.

Laminin-8 and laminin-10 are secreted by endothelial cells, and are major components of the subendothelial basement membrane (Sorokin, L.M. et al., Dev. Biol. 189(2), 285-300 (1997); Iivanainen, A. et al., J. Biol. Chem. 272(44), 27862-8 (1997); Patton, B. L. et al., J. Cell Biol. 139(6), 1507-21 (1997), Miner, J.H. et al., J. Cell Biol. 137(3), 685-701 (1997); Sorokin, L. et al., Eur. J. Biochem. 223(2), 603-10 (1994); and

Tokida, Y. et al., J. Biol. Chem. 265(30), 18123-9 (1990)). The data presented below demonstrates that isolated laminin 10 promotes endothelial cell attachment and migration.

Thus, in one embodiment the isolated laminin 10 is used to promote re-endothelialization, and to thus inhibit abnormal smooth muscle cell proliferation, at the site of a vascular injury. In another embodiment, isolated laminin 10 is coated onto grafts to improve the "take" of grafts. As used herein the term "graft" refers to both natural and prosthetic grafts and implants.

In a further aspect, the present invention comprises medical devices with improved biocompatibility, wherein the devices are coated with isolated laminin 10 or pharmaceutical compositions thereof, alone or in combination with other proteins or agents that serve to increase the biocompatibility of the device surface. The coated device stimulates cell attachment (such as endothelial cell attachment), and provides for diminished inflammation and/or infection at the site of entry of the appliance.

Such medical devices can be of any material used for implantation into the body, and preferably are made of or coated with a biocompatible metal that may be either stainless steel or titanium. Alternatively, the device is made of or coated with a ceramic material, or a polymer including but not limited to polyester, polyglycolic acid or a polygalactose-polyglycolic acid copolymer.

One particular use of the present invention is to increase cell adhesion to target surfaces, including but not limited to endothelial cell adhesion. For example, vascular grafts and stents may be coated with isolated laminin 10 or pharmaceutical compositions thereof to stimulate endothelial cell attachment.

If the device is made of a natural or synthetic biodegradable material in the form of a mesh, sheet or fabric, isolated laminin 10 or pharmaceutical compositions thereof may be applied directly to the surface thereof. Appropriate cells may then be cultured on the matrix to form transplantable or implantable devices, including dental abutment pieces, needles, metal pins or rods, indwelling catheters, colostomy tubes, surgical meshes and any other appliance for which coating with isolated laminin 10 is desirable. Alternatively, the devices may be implanted and cells may be permitted to attach in vivo.

Coupling of the isolated laminin 10 may be non-covalent (such as by adsorption), or by covalent means. The device may be immersed in, incubated in, or sprayed with the isolated laminin 10 or pharmaceutical compositions thereof.

The dosage regimen for various treatments using the isolated laminin 10 of the present invention is based on a variety of factors, including the type of injury or condition, the age, weight, sex, medical condition of the individual, the severity of the condition, and the route of administration. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Laminins are extremely potent molecules, and one or a few molecules per cell could produce an effect. Thus, effective doses in the pico-gram per milliliter range are possible if the delivery is optimized. Laminins are sometimes present in an insoluble form in the basement membrane and have the capability of polymerizing at concentrations as low as about 50 μg/ml, depending on the laminin isoform and the conditions. Laminins can also polymerize into a gel at a concentration of about 2-3 mg/ml. Dosage levels of the order of between 1 ng/ml and 10 mg/ml are thus useful for all methods disclosed herein, preferably between about 1 μg/ml and about 3 mg/ml.

The present invention also provides a method for inducing cell attachment to the device (as disclosed above), comprising coating the appliance with isolated laminin 10 or pharmaceutical compositions thereof prior to incubation with cells appropriate for the desired application.

In another aspect of the present invention, isolated laminin 10 is used for the culture of cells, including but not limited to endothelial cells, by contacting the cells with an amount effective of isolated laminin 10 to stimulate cell attachment and subsequent cell stasis, proliferation, differentiation, and/or migration. The isolated laminin 10 can either be provided in the cell culture medium, or as a cell culture medium supplement, or may be coated on the surface of a cell growth substrate. In a preferred embodiment, the method further includes contacting the cells with other compounds, including but not limited to any of the collagens, other laminin types, fibronectin, α-dystroglycan, cadherins, integrins, entactin/nidogen, α-dystroglycan, glycoproteins, proteoglycans, heparan sulfate proteoglycan, glycosaminoglycans, epidermal growth factor or nerve

growth factors, vascular endothelial growth factor, fibroblast growth factor, and peptide fragments thereof.

The cells may comprise primary cells or cell culture cell lines. The methods of this aspect of the invention can be used in vivo, or in vitro.

In a preferred embodiment, isolated laminin 10 is used to coat the surface of a substrate, to promote cell adhesion to the substrate. The substrate used herein may be any desired substrate. For laboratory use, the substrate may be as simple as glass or plastic. For use in vivo, the substrate may be any biologically compatible material capable of supporting cell adhesion. Suitable substrate materials include shaped articles made of or coated with such materials as collagen, regenerated collagen, polyglycolic acid, polygalactose, polylactic acid or derivatives thereof; biocompatible metals such as titanium and stainless steel; ceramic materials including prosthetic material such as hydroxylapatite; synthetic polymers including polyesters and nylons; polystyrene; polyacrylates; polytetrafluoroethylene and virtually any other material to which biological molecules can readily adhere. The determination of the ability of a particular material to support adhesion of the isolated laminin 10 of the invention requires only routine experimentation by the skilled artisan.

In a further aspect, the present invention provides cell growth substrates for adhesion and culturing of cells, by providing an amount effective of isolated laminin 10 for the attachment of cells to a cell culture device. The substrates may comprise any of the substrates discussed above.

In another aspect of the present invention, an improved cell culture medium is provided, wherein the improvement comprises addition to the cell culture medium of an effective amount of isolated laminin 10 to the cell culture medium to promote the adherence, proliferation, and/or maintenance of cells. Any cell culture media that can support the growth of cells can be used with the present invention. Such cell culture media include, but are not limited to Basal Media Eagle, Dulbecco's Modified Eagle Medium, Iscove's Modified Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, F-10 Nutrient Mixtures, Opti-MEM® Reduced-Serum Medium, RPMI Medium, and Macrophage-SFM Medium or combinations thereof.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as either a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement. Culture media is commercially available from many sources, such as GIBCO BRL (Gaithersburg, MD) and Sigma (St. Louis, MO).In an alternative embodiment, the laminin 10 is used as a cell culture supplement.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Examples

Cloning of the Human Laminin α5 cDNA

The previously published mouse laminin α5 sequence (SEQ ID NO.:3) was used to search EST-databases. Based upon sequences of the identified ESTs, oligonucleotide primers were synthesized and used for PCR amplification of several human α5 specific probes with λgt11 cDNA library (Clontech) as template. These probes were used for screening of λgt11 cDNA libraries (Clontech) from human lung, fetal lung and fetal kidney. This resulted in the isolation of several clones, and further screening was performed with PCR amplified selected regions of these clones. This walk generated clones covering 2134 base pair of coding sequence and 195 base pairs of 3'UTR in the C-terminal part, and 5354 base pairs in the N-terminal part, but lacking a translation initiation start site. The center part, comprising base pairs 5582-9316, was obtained by PCR amplification from a Human Lung MARATHON READY™ cDNA-mix (Clontech). The remaining 296 base pairs of coding sequence and a 67 base pair 5'UTR end was obtained with SMART™ RACE cDNA Amplification Kit (Clontech) using poly-A RNA purified with QuickPrep mRNA Purification Kit (Pharmacia Biotech) from HEK293 cell lysate. The reverse transcription was performed with the MMLV reverse transcriptase SuperscriptII (Life Technologies), and subsequent PCR amplification was performed with Advantage®-GC 2 PCR kit (Clontech). This 363 base pair N-terminal

sequence was confirmed by sequencing genomic P1-clone (GenomeSystems) obtained by screening with a PCR generated probe from nucleotides 344-452. This generated a full-length sequence, but most of the sequence was only covered by a single λ-clone or PCR fragment. To further confirm the sequence, we used PCR amplification of SMART™RACE-generated cDNA-mixes from HEK293 cells, human placenta total RNA and from Human Lung Marathon Ready™ cDNA-mix. This generated new clones so that all regions of the cDNA were covered by more than one clone from different sources. In the case of suspected polymorphisms, several clones from different sources were compared. Sequencing was performed on an ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer) using ABI PRISM®BigDye™ Terminator Cycle Sequencing kit (PE Applied Biosystems). Sequence analysis was performed with AutoAssembler™ (PE Applied Biosystems) and sequence comparative analysis with the GCG-software (Group, G.C. et al., *Program for the GCG* Package, Version 10.1, Genetics Computer Group, Madison, WI (2000)).

Expression Constructs

For expression of the human laminin α5 chain containing a C-terminal FLAG epitope, the full-length cDNA was constructed as follows. To obtain overlapping cDNA clones, PCR amplification of SMART™ RACE-generated cDNA-mixes from HEK293 cells, human placenta total RNA, and Human Lung MARATHON READψ™ cDNA-mix was performed using ADVANTAGE®-GC 2 PCR kit and Pfu Turbo polymerase (Boeringer-Mannheim). All PCR derived cDNA fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced (AmpliTag FS on an ABI310 sequencer, Perkin-Elmer) to ensure that no mutations had occurred during amplification. All primers for PCR and pCR2.1-TOPO™ plasmids into which the PCR derived cDNA fragments were cloned are shown in Table I. To ensure efficient and correct translation initiation, the Kozak sequence (accgcc, (Kozak, M., *J. Cell Biol.* 115(4), 997-903 (1991)) was edited to match the consensus. Primer KZK1 contained modified Kozak sequence and primer FLAG1 contained the FLAG sequence encoding the FLAG epitope (N-Asp-Tyr-Lys-Asp-Asp- Asp-Asp-Lys-C). (SEQ ID NO:37)

The EcoRI-BamHI insert from KBX3 was cloned into EcoRI-BamHI digested pUC18 vector to make KBX4. The BamHI-XbaI fragment from BBL3 was ligated into BamHI-XbaI sites of KBX4 to make KBY1'. The BNK2' BclI-NotI fragment was cloned into KBY1' BclI-NotI sites to make KNX3. The 1.2 kilobase Bpu10I-NotI fragment of KNX3, which contained unwanted mutation was corrected by replacing the mutated fragment with non-mutated Bpu10I-NotI fragment from BNL12 to make KNX4'. The 1.8 kilobase BbvCI-SalI fragment of NSK5 was replaced by D29D30III BbvCI-SalI fragment to make NSX1. The NotI-SalI fragment from NSX1 and AscI-EcoRI fragment containing FLAG epitope from SFK2 were cloned into SFL12 to make NFX4. The 5.3 kilobase NotI-HindIII fragment from NFX4 was ligated into NotI-HindIII sites of KNX4' to make KFX5 with full-length cDNA. The final expression construct named HLN5Full.pcDNA was made by inserting the KFX5 EcoRI fragment into the EcoRI sites of pcDNA3.1/Zeo(-) mammalian expression vector (Invitrogen) in correct orientation. The construct used for expression of human laminin β1 was constructed from a baculovirus expression vector (Pikkarainen, T. et al., *Eur. J. Biochem.* 209(2), 571-82 (1992)) by ligation of the insert into pIRES-vector (Clontech). The construct used for expression of laminin γ1 (HG1) has been described previously (Kortesmaa, J. et al., *J. Biol. Chem.* 275(20), 14853-9 (2000)).

Antibodies and Control Proteins

Anti-laminin α5 (15H5, (Kikkawa, Y. et al., *J. Bio. Chem.* 273(25), 15854-9 (1998)) monoclonal antibody (mAb) was kindly provided by Dr. K. Sekiguchi. Anti-laminin β1 (DG10,Virtanen, I. et al., Am. J. Pathol. 150(4), 1421-31 (1997)) mAb was kindly provided by Dr. I. Virtanen. Anti-laminin β1 (2E8, Engvall, E. et al., J. Cell Biol. 103(6 Pt 1), 2457-65 (1986)) mAb was kindly provided by Dr. E. Engvall. Anti-FLAG M2 mAb, purified control mouse IgG, collagen type I from calfskin, collagen type IV (Col IV) from mouse-EHS- tumor and heparin (grade I-A) were purchased from Sigma. Anti-laminin γ1 (clone 22) mAb was purchased from Transduction Laboratories. Mouse mAb against integrin α6 (BQ16) was purchased from Alexis Biochemicals. Mouse function blocking mAbs against integrin α1 (FB12), α2 (P1E6), α3 (P1B5), αv (NKI-M9), αϖβ3 (ΛM609), mouse mAb against integrin β4 (ASC-3) and control rat IgG2a

were obtained from Chemicon. Rat function blocking mAbs against integrin α6 (GoH3), mouse function blocking mAbs against integrin β1 (4B4) and mouse mAbs against integrin α4 (HP2/1), αv (AMF-7), and β3 (SZ-21) were obtained from Coulter. Secondary Ab conjugates anti-mouse IgG-horseradish peroxidase (HRP) and FITC-conjugated F(ab)2 fragments of rabbit anti-mouse immunoglobulin were purchased from Dako. RGDS-peptide, cyclical RGDS-peptide, control RAGS-peptide, mouse mAb against integrin α5 (P1D6) and human vitronectin from plasma were purchased from Life Technologies. Human fibronectin (FN) from plasma was obtained from Roche. EHS-derived laminin-1/nidogen complex (laminin-1/Nd) was kindly provided by Dr. J. Engel.

Production and Purification of Recombinant Laminin-10

r-laminin-10 was produced in human embryonic kidney cells (HEK293, ATCC CRL-1573) cultured in DMEM, pyruvate, 10% FCS in humidified 5% CO_2 atmosphere at 37°C. Wild-type cells were transfected using the standard calcium-phosphate method with the HG1 construct and stable colonies were selected using 100 mg/ml hygromycin (Cayla). All further cell culture and clonal expansion was carried out in continuous presence of relevant selection antibiotics. A highly expressing clone was then transfected with the human laminin β1 construct and stable clones were selected using 500 mg/ml G418 (Life Technologies). A clone highly expressing both laminin γ1 and laminin β1 was finally transfected with HLN5Full.pcDNA and stable colonies were selected using 200 mg/ml zeocin (Cayla). The clones showing the highest secretion were expanded further.

For production of r-laminin-10, confluent cells were cultured in DMEM supplemented with 1mM pyruvate and insulin-transferrin-selen supplement (Sigma) for up to five days. r-laminin-10 was affinity purified using anti-FLAG M2 matrix (Sigma). The collected medium was incubated in batch mode with the matrix overnight at 4°C with agitation. Bound r-laminin-10 was competitively eluted with 50 mg/ml FLAG peptide (Sigma) in TBS/E (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA) at room temperature. The elute was concentrated and the buffer was replaced by PBS using 30 kD cut-off ultrafiltration (Millipore). Finally the concentrated solution was passed

through 0.2 mm filter to remove self-aggregated polymers. Recombinant human laminin-8 (r-laminin -8) was produced in HEK293 cells and isolated using anti-FLAG matrix and ion-exchange chromatography.

Characterization of Recombinant Laminin-10

Secreted laminin in medium and after purification was characterized using 5% SDS-PAGE and 4-15% gradient SDS-PAGE. Proteins were visualized using silver staining or transferred onto PVDF. The membranes were probed with mAbs described above. After washing, the membranes were incubated with HRP-conjugated goat anti-mouse antibody. The immunoreactivity was detected by a chemiluminescent kit (Life Science Products) according to the manufacturer's instructions.

Electron microscopy was performed by the rotary shadowing technique as described previously (Engel, J., Methods Enzymol. 245, 469-88 (1994)). Briefly, protein (25-50 mg/ml) in 0.2 M ammonium bicarbonate, pH 7.4, or 0.1 M acetic acid was mixed with an equal volume of glycerol and sprayed onto freshly cleaved mica discs. These were dried in high vacuum, shadowed with platinum/carbon at an angle of 9° and replicated. Negative staining was performed at neutral pH in order to avoid dissociation of aggregates by the acid pH in the routine procedure. Ten ml of a solution in PBS was put on a glow discharged collodium and carbon grid and 5 ml of a 2% sodium phosphotungstate solution of pH 7 was added. After removal of the first stain, incubation was repeated for 2 min.

Cell Culture and HSVEC isolation

Human fibrosarcoma HT-1080 (CCL-121) cells were from ATCC. Immortomouse brain capillary endothelial (Kanda, S. et al Exp. Cell Res. 248(1), 203-13 (1999)) cells were kindly provided by Dr. L. Claesson-Welsh. All cells were cultured in humidified 5% CO_2 atmosphere. HT-1080 cells were cultured in DMEM, 10% FCS, pyruvate at 37°C. IBE cells were cultured in F-12, 10% FCS, 2 units/ml γ-interferon on gelatin-coated plastic at 33°C. Prior to assay, the IBE cells were cultured in serum-free F-12 at 37°C without γ-interferon for 24 hours.

As approved by the ethical committee at the Karolinska Hospital, Stockholm, Sweden, residual segments of the great saphenous vein were collected from patients undergoing coronary bypass surgery. HSVECs were isolated as previously described (Haegerstrand, A. et al., J. Vasc. Surg. 16(2), 280-5 (1992)). Briefly, veins were rinsed with MEM (Life Technologies) and filled with 0.1% collagenase and 0.16% dispase (Boehringer Mannheim) in MEM for 20 min at 37°C in an 8% CO2-humidified atmosphere. Cells were cultured in MEM containing 40% heat-inactivated pooled human serum (HS), 1 nmol/L choleratoxin (CT; Sigma), 33 mmol/L isobutylmethylxantine (IBMX; Sigma), and antibiotics. HSVECs were seeded on gelatin-coated plates and passaged (1:3). HSVECs were characterized with monoclonal anti-human von Willebrand factor-related antigen (Dako) and HSVECs between passages 4 and 7 were used in the experiments.

Cell Adhesion Assays

Adhesion assay was performed as described previously (Kortesmaa, J. et al., J. Biol. Chem. 275(20), 14853-9 (2000)). Briefly, 96-well plates (Maxi-Sorp, Nunc) were coated with proteins overnight at 4°C. The remaining protein binding capacity was saturated by addition of 2% heat-inactivated BSA in PBS.

For the assay, cells were suspended in buffered serum-free medium at $3x10^4$ cells/well. DMEM, 25mM Hepes, pyruvate was used for HT-1080 cells, F-12, 25 mM Hepes, 0.25% BSA for others. Antibodies or other test compounds were added to the cell suspension and the cells were allowed to recover at 37°C for 30 min. Integrin mAbs were used at 10 mg/ml, RGD-peptides at 0.25 mg/ml, heparin at 5 mg/ml, and EDTA at 5mM. The cells were then allowed to adhere for 60 min at 37°C. Bound cells were quantitated by crystal violet staining. None of the cell lines bound appreciably to BSA. When the quantitative results were calculated, binding to BSA was given a value of zero, while the relevant control was given the value 100. The mean and standard deviation (S.D.) were calculated from results obtained from parallel wells.

Immunofluorescence Flow Cytometry

Briefly, suspended HSVECs were incubated in PBS containing anti-integrin mAbs against α1-6, αv, β1, β3, and β4 for 30 min at 4°C. Following washing, cells were incubated with FITC-conjugated F(ab)2 fragments of rabbit anti-mouse immunoglobulin for 30 min at 4°C. Cells were then analyzed in a FACS can flow cytometer (Becton Dickinson). Mouse IgG was used as a negative control.

Cell Migration Assays

Cell migration assay was performed as described previously (Jansson, K. et al., Eur. J. Vasc. Endovasc. Surg. 16(4), 334-41 (1998)). Flat-bottom 24-well culture plates (Corning) were coated with proteins overnight at 4°C. The remaining protein binding capacity was saturated by addition of 2% heat-inactivated BSA in PBS. Thereafter a 4x10 mm stainless steel-weight was put on the center of well, before seeding HSVECs at $2x10^5$ cells/well. After adhesion for 2 days in MEM with 30% HS, the steel-weight was removed. A gap devoid of HSVECs was thus created, with two broad (10 mm) EC-edges facing each other at a distance of 4 mm. During endothelialization, HSVECs were incubated in MEM with 40% HS, CT and IBMX for 2 days after removing the steel-weight. The cells were visualized by 0.1% crystal violet staining.

Results

Sequence of Human Laminin Alpha 5 Chain

The full-length laminin α5 cDNA coding sequence (Fig. 1) (SEQ ID NO:1) consisted of 11,088 base pair with an open reading frame encoding 3696 amino acids (SEQ ID NO.:2). Compared to the previously reported mouse laminin α5 sequence (GENBANK™ accession number U37501; Miner, J.H. et al., J. Biol. Chem. 270(48), 28523-6 (1995)) (SEQ ID NO.:4), we obtained an additional 79 amino acids in the N-terminal end. The mouse sequence has an additional stretch of 20 amino acids in the C-terminus, compared to the human sequence. Alignment of mouse and human laminin LG5-modules with other published sequences (not shown) revealed similar C-terminal length in all cases except for the mouse α5. Comparison with the mouse laminin α5 showed an overall amino-acid identity of 79%. The previously reported adhesive

tripeptide sequence LRE (Hunter, D.D. et al., Cell 59(5), 905-13 (1989)) was not conserved in the human chain (amino acid residues; 3176-3178 LQQ), while the two RGD-sequences were conserved (Fig. 1) . The human sequence contained two extra cysteines (amino acid residues; 3173 and 3663) and a hinge region between LG 3 and LG 4 that is seven residues longer than the mouse sequence. In addition, there was a stretch of four extra amino acids in domain IV (amino acid residues; 1680-1683) in the human sequence. We generated cDNAs from four different sources (placenta, HEK293 cells, and lung-marathon-ready-cDNA from two sources) and thereby detected four possible polymorphisms in domain IIIa; 5698:A-G (1900: Met-Val), 5722: G-A (1908: Ala-Thr), 6158: G-A (2053: Arg-Thr), 6184: A-G (2062: Asn-Asp) and one in the G domain; 9235:T-C (3079: Trp-Arg). The amino acids chosen for the r-laminin-10 construct at these possible polymorphic sites are those shown in Fig. 1.

Production and Characterization of Recombinant laminin-10

Conditioned medium from wild-type HEK293 cells did not react in western blotting with the anti-laminin α5, anti-laminin β1, anti-laminin γ1, or anti-FLAG Abs, indicating that these cells express endogenous laminins at very low amounts if at all (data not shown). After triple transfection, the best cell clone produced 2-3 mg of r-laminin-10 per liter of medium, which is quite high considering the size and complexity of the protein.

Immunoaffinity purification with anti-FLAG M2 matrix followed by competitive elution with FLAG-peptide resulted in highly purified protein as seen in silver stained SDS-PAGE gels (Fig 2a). Under reducing conditions, two bands were seen, a 400 kD band corresponding to the laminin α5 chain and a 200 kD band corresponding to the laminin β1 and γ1 chains, which have similar molecular weights (Fig. 2a). In western blotting of the conditioned medium, two bands of approximately 350 and 400 kD could be seen with the laminin α5 mAb (Fig. 2b). The anti-FLAG antibody reacted with a 400 kD and a 40 kD fragment (Fig 2b and not shown). Taken together, these data indicate that the 400 kD fragment is the intact laminin α5, the 350 kD is a N-terminal fragment and the 40 kD is a C-terminal fragment harboring the FLAG epitope. Under non-reducing conditions, most of the protein appeared at the top of the gel as a very high molecular

weight band, which was immunoreactive with α5, FLAG, β1 and γ1 mAbs, showing that the r-laminin-10 was produced as disulfide-crosslinked heterotrimer (Fig. 2c). A minor band of approximately 400 kD was also seen in silver staining and in western blotting with α5, FLAG, β1 and γ1 mAbs. The non-covalently associated α5 chain had an apparent molecular weight similar to the β1/γ1 dimer, which explains the immunoreactivity of the minor band with α5 and FLAG mAbs.

Rotary shadowing EM revealed the r-laminin-10 protein as having three short arms and one long arm in accordance with the expected structure. Some monomers were shown to have an elongated globular domain in one of the short arms, which could be domain IVb. Oligomers dominated the preparation.

Cell Binding to r-laminin-10

Vascular endothelial cells undergo drastic morphological and functional changes during angiogenesis, and it is well established that the behavior of the cell is critically influenced by its interaction with components of the extracellular matrix. Because of this fact, endothelial cell attachment and migration on grafts used in vascular surgery might be improved if the surfaces of these non-biological materials would be pre-coated with ECM proteins, e. g. laminins. The ingrowth of endothelial cells on the surfaces of grafts, a process known as endothelialization, has been shown to be of critical importance for preventing thrombus formation on the graft material, and for reducing neointimal hyperplasia. Many adhesive substrate coatings to enhance endothelial cell attachment have been tested (Dixit, P., et al. (2001) *J. Biomed. Mater. Res.* 56(4), 545-55), but the long term patency of small-diameter vascular grafts is still disappointing, primarily due to stenosis and thrombus formation (Pevec, W. C., et al. (1992) *J. Vasc. Surg.* 16(1), 60-5; Watelet, J., et al. (1997) *Ann. Vasc. Surg.* 11(5), 510-9).

To investigate the biological activity of r-laminin-10, we assayed it for cell adhesion properties. Cell adhesion onto the laminin coated substratum is mediated predominantly by the integrin family of adhesion receptors. Several integrins have been implicated as receptors for laminin-10 or laminin-10/ 11, including α2β1, α3β1, α6β1, and α6β4 (Kikkawa, Y. et al., J. Biol. Chem. 273(25), 15854-9 (1998); Kikkawa, Y. et al., J. Cell Sci. 113(Pt 5), 869-76 (2000); Ferletta, M. et al., J. Cell Sci. 112 (Pt 1), 1-10

(1999); Gu, Y. et al., Blood 93(8), 2533-42 (1999); Pouliot, N. et al., Exp. Cell Res. 261(2), 360-71 (2000); and Tani, T. et al., Exp. Cell Res. 248(1), 115-21 (1999)). In addition, Nielsen and co-workers recently demonstrated that domain IV of the laminin α5 chain is a binding site for integrin α2β1, α3β1, α4β1, and α6β1 (Nielsen, P.K. et al., J. Biol. Chem. 276(14), 10906-12 (2001)). As a general model, we used the HT-1080 fibrosarcoma cell line, which expresses a wide variety of integrin receptors such as α2, α3, α5, α6 and β1 (Wayner, E.A. et al., J. Cell Biol. 121(5), 1141-52 (1993)). Different blocking anti-integrin antibodies were used to identify the integrin receptors mediating cell binding to r-laminin-10. Two endothelial cell types were also studied: HSVECs were used as model for macrovascular endothelial cells and IBE cells for microvascular endothelial cells. HT-1080 cells adhered to fibronectin equally strongly as to r-laminin-10 (Fig. 3a) while laminin-1 and r-laminin-8 were less effective in promoting cell adhesion (Fig. 3a). Similar results were obtained with IBE cells and HSVECs (Fig. 3b and c). Based on these results, further experiments with blocking integrin mAbs were performed using a coating concentration of 10 mg/ml.

Monoclonal Abs against either α3 or β1 inhibited HT-1080 cell binding to r-laminin-10 by approximately 80%, indicating that integrin α3β1 was a major mediator of adhesion to r-laminin-10 (Fig. 4). In addition, mAbs against integrin α2 had partial inhibitory effect on adhesion to r-laminin-10 (Fig. 4). Since some adhesion remained after blocking of the integrin β1, other receptor classes besides β1 could be involved in the cell adhesion. Monoclonal Abs against integrins α6 and αv had no effects on the adhesion of HT-1080 cells to r-laminin-10 either alone (Fig. 4) or in various combinations (α3+α6, β1+α6, β1+αvβ3, β1+α6+αvβ3, β1+α6+αv; not shown). This indicates that α6 integrins (α6β1, α6β4) or αv integrins (αvβ1, αvβ3 or αvβ5) were not mediating the cell adhesion.

HSVEC binding to the r-laminin-10 was also studied. To determine which integrins were present on the cell surface, we performed fluorescent cell sorting assay using mAbs against integrin subunits α1, α2, α3, α4, α5, α6, αv, β1, β3 and β4. From these results it can be concluded that HSVEC express large amounts of α2β1, α5β1 and αvβ3, moderate amounts of α3β1, and small amounts of α1β1, α6β1 and α6β4, but integrin α4β1 was not detected. The HSVEC binding was most efficiently inhibited by

mAbs against integrin α3 and β1, but also against α2 (Fig. 3) had partial effect, in a fashion similar to that observed for HT-1080 cells. Integrin α6 was only weakly expressed on HSVECs and, consequently, mAbs against this integrin did not inhibit binding to r-laminin-10.

Cell adhesion to r-laminin-10 was found to be dependent of divalent cations since it could be abolished by 5 mM EDTA in both HT-1080 cells and HSVECs (Fig.3). Heparin, when used at 5 mg/ml, had no effect on the adhesion of either cell type (Fig. 3). Since the α5-chain has conserved RGD-sequences, we tested the effect of RGD peptides, which are reported to block the function of various RGD-dependent integrins (such as α5β1 and the αv family) (Pierschbacher, M.D. et al., Nature 309(5963), 30-3 (1984)). Neither linear nor cyclic RGD-peptides had any effect at 0.25 mg/ml concentration on adhesion of either HT-1080 cells or HSVECs to r-laminin-10 (Fig. 4). It was, furthermore, observed that the cell binding activity of r-laminin-10 was sensitive to air-drying, as we have previously reported for r-laminin-8 (Kortesmaa, J. et al., J. Biol. Chem. 275(20), 14853-9 (2000)). When the coated protein was allowed to air dry for 20 min at room temperature before adding the cells, the cell binding activity of r-laminin-10 was completely lost (data not shown).

Cell Migration

Laminins have been shown to stimulate cell migration during development, and in many pathological processes. We examined the ability to promote HSVEC migration on dishes coated with 10 mg/ml of r-laminin-10 or other adhesive proteins. The migration assay was repeated three times using HSVEC obtained from three different donors. Among the seven different adhesive proteins examined, r-laminin-10 was the most potent in promoting HSVEC migration *in vitro*. In addition to r-laminin-10, type IV collagen was also quite potent in promoting HSVEC migration. Laminin N-1 and gelatin were of roughly equal potency but significantly lower than r-laminin-10, and r-laminin-8 was the least potent among the proteins examined.

Discussion

Several cell types have been tested for identification of the integrin receptors for laminin-10, but no report exists concerning endothelial cells. In this study, we demonstrated that HSVECs use integrins α2β1 and α3β1 to mediate cell adhesion to r laminin-10, and similar results were obtained for HT-1080 cells. An antibody against the integrin α6 subunit, either alone or combination with other mAbs (α3+α6, β1+α6, β1+α6+αvβ3, β1+α6+αv), did not inhibit cell adhesion (Fig.4 and not shown), indicating that integrin α6 is not an important receptor in these cells for r laminin-10, although the α6 integrins have previously been implicated as receptors for laminins in general and laminin-10 in particular (Kikkawa, Y. et al., J. Cell Sci. 113(Pt 5), 869-76 (2000)).

HSVECs, as well as HT-1080 and IBE cells, attached to r-10 more strongly than to laminin-1 and r laminin-8. Poor adhesion of HSVECs to r laminin-8 is not surprising considering the fluorescent cell sorting data showing that the cells have little α6 integrins, which have previously been shown to be receptors for r-laminin-8 (Kortesmaa, J. et al., J. Biol. Chem. 275(20), 14853-9 (2000)). We can, therefore, conclude that the integrin-binding is distinctly different between the two main forms of endothelial laminins, as well as between different endothelial cell types, as we have previously shown that IBE and bovine capillary endothelial cell adhesion onto r-laminin-8 is mediated predominantly by α6 integrins (Kortesmaa, J. et al., J. Biol. Chem. 275(20), 14853-9 (2000)).

Cell migration-promoting activities of different laminins appear to be dependent on cell specific factors. Human glioblastoma cell line T98G showed best migration on laminin-8 (Fujiwara, H. et al., J. Biol. Chem. 276(20), 17550-8 (2001)) compared to laminin-2/4, laminin-5, laminin-10/11 and fibronectin, while LIM1215 carcinoma cells migrate more efficiently on laminin-10 than on collagen type I, type IV or laminin-1 (Pouliot, N. et al., Exp. Cell Res. 266(1), 1-10 (2001)). Here, we demonstrated that r-laminin 10 was the most potent matrix of the components tested in promoting endothelial cell (HSVEC) migration *in vitro* (Fig. 5). Interestingly, HSVEC adhesion to commercial laminin-10/11 and to r-laminin-10 was equally strong, but the potency of laminin-10/11 in promoting HSVEC migration was much lower than that of r-laminin-10.